*ANALYSIS OF THE 5′-TERMINAL NUCLEOTIDE SEQUENCES OF RIBONUCLEIC ACIDS, II. COMPARISON OF THE 5′-TERMINAL NUCLEOTIDE SEQUENCES OF RIBOSOMAL RNA'S FROM DIFFERENT ORGANISMS**

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The use of end-group analysis is valuable for determining the homogeneity and molecular weight of macromolecules. The synthesis of RNA upon a DNA template proceeds from the 5′- to the 3′-end of the RNA sequence, and the identification of the 5′-termini of RNA molecules is therefore of particular importance in characterizing them. With this in view, a procedure for analyzing 5′-terminal nucleotide sequences of large RNA molecules was developed by one of us.[1] By this technique, it was found that two ribosomal RNA's present in *Escherichia coli* had unique sequences at their 5′-termini. A logical next step was to compare these sequences with those of ribosomal RNA's from other organisms. According to Midgley[2] and Miura,[3] the ribosomal RNA of bacteria is quite constant in nucleotide composition, in contrast to DNA, which varies widely in composition from species to species. When tested with cell-free amino acid-incorporating systems, ribosomes from various bacterial species have been found to be interchangeable.[4, 5] Accordingly, ribosomal RNA's were prepared from four species of bacteria with GC/AT ratios in DNA ranging from 0.53 (*Bacillus cereus*) to 2.33 (*Sarcina lutea*), and from one species of yeast, and their 5′-termini were analyzed. The results obtained are described in this communication.

Materials and Methods.—Organisms: The bacterial and yeast species with GC/AT ratios of their DNA and the nucleotide composition of their RNA's are listed in Table 1.

Culture media and harvesting cells: Culture media were as follows: *S. lutea* and *B. stearothermophilus*—10 gm polypeptone, 2 gm yeast extract, and 8 gm NaCl in 1 liter of water, pH 7.4; *B. cereus*—4 gm glucose, 1 gm NH_4Cl, 120 mg $MgSO_4$, 160 μg $FeCl_3$, and 2 gm casamino acids in 1 liter of 0.05 *M* Tris-HCl, pH 7.4; *B. subtilis*—1 gm Na-citrate, 2 gm $(NH_4)_2SO_4$, 5 gm casamino acids, 120 mg $MgSO_4$, 5 gm sucrose in 1 liter of 0.05 *M* phosphate buffer, pH 7.4; yeast—5 gm yeast extract, 4 gm $(NH_4)_2SO_4$, 0.25 gm $MgSO_4$, 0.25 gm $CaCl_2$, 5 gm casamino acids, 5 gm peptone, and 40 gm glucose in 1 liter of water, pH 5.0. All bacteria were grown with aeration at 37°C, except *B. stearothermophilus*, which was grown at 60°C. Yeast was cultured at 30°C. Cells were harvested during the logarithmic phase (OD_{650} = 0.6–0.8) and stored in a freezer until use.

TABLE 1

COMPARISON OF THE GC/AT RATIOS OF DNA AND THE NUCLEOTIDE COMPOSITIONS OF RIBOSOMAL RNA

Organism	GC/AT of DNA	Nucleotide Composition of Ribosomal RNA (A = 1.00) C	A	G	U
Bacillus cereus	0.53[9]	0.87	1.00	1.26	0.84[3]
Bacillus subtilis	0.74[9]	0.93	1.00	1.31	0.83[3]
Bacillus stearothermophilus	0.88[10]	0.85	1.00	1.33	0.68[11]
Escherichia coli	1.00[9]	0.96	1.00	1.36	0.87[3]
Sarcina lutea	2.33[9]	1.05	1.00	1.59	1.01[3]
Saccharomyces cerevisiae	0.56[9]	0.71	1.00	1.04	0.93[2]

Data were taken from the references as noted.

Ribosomal RNA: Ribosomal RNA was prepared directly from the frozen cells by the method (B) described in the previous paper[1] and the light and heavy RNA components (16*S* and 23*S* RNA's of bacteria and 18*S* and 28*S* RNA's of yeast) were separated by sucrose density gradient centrifugation.

Enzymes: A mutant strain, constitutive in alkaline phosphatase, was isolated from *E. coli* A19, from which alkaline phosphatase was purified by the method described in the previous paper.[1] The procedures for purification of RNase-free polynucleotide kinase were as in the previous paper,[1] except that the final phosphocellulose step was omitted and the DEAE-cellulose column chromatography was repeated twice.

Alkaline phosphatase treatment of RNA: The procedure was as described previously,[1] except that twofold units of enzyme were added to the reaction mixture.

*Phosphorylation of RNA with radioactive orthophosphate (*P):* The detailed procedures for labeling the phosphatase-pretreated RNA with *P and isolation of RNA phosphorylated with *P(abbreviated as 5′(*P)-RNA) from the reaction mixture were described previously.[1] The preparation of (γP^{32})ATP containing about 10^9 to 10^{10} cpm/μmole was as described by Glynn and Chappell.[7]

*Analysis of radioactive fragments produced by hydrolysis of 5′(*P)-RNA:* The procedures for hydrolysis of 5′(*P)-RNA with alkali, T1 RNase, and pancreatic RNase, and for separation of the resulting radioactive fragments by DEAE-Sephadex and Dowex 1X2 column chromatography were previously described.[1] The heavy RNA component of yeast produced *pUpXpGp (X = C, U, or A), and the second base was identified by the following procedures. Three trinucleotides—UpCpGp, UpApGp, and UpUpGp—were prepared from a T1-RNase hydrolysate of ribosomal RNA and their 5′-hydroxyl groups were phosphorylated with *P, using polynucleotide kinase. The resulting *pUpCpGp, *pUpApGp, and *pUpUpGp were chromatographed on Dowex 1X2 columns (0.6 cm × 25 cm) (minus 400 mesh) together with four nonradioactive marker nucleoside triphosphates. Elution was with a linear gradient from 0.01 *N* HCl to 0.01 *N* HCl–0.5 *M* NaCl (total 500 ml). The three trinucleoside tetraphosphates were clearly separated by this chromatographic procedure. Samples from the hydrolysis of ribosomal RNA were chromatographed under similar conditions to identify the sequences by referring to the chromatographic positions thus determined.

Results.—The ribosomal RNA preparations from the five organisms were all found to be very poor substrates for polynucleotide kinase. The phosphorylation levels at the 5′-termini increased markedly only when the RNA's were pretreated with alkaline phosphatase. The results of a typical experiment with ribosomal

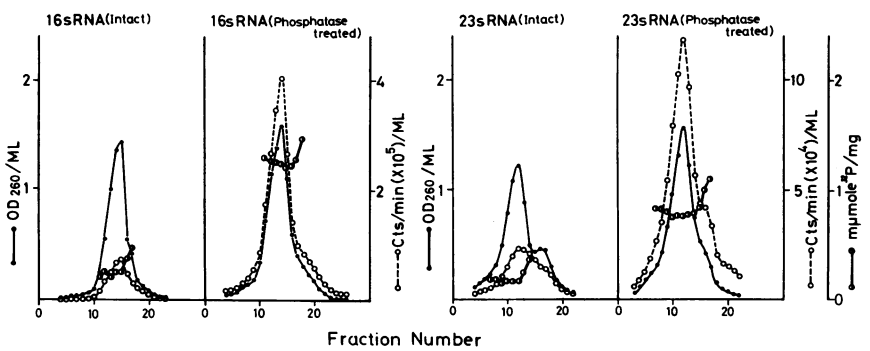

FIG. 1.—Acceptance of P^{32} from (γP^{32})-ATP by intact or phosphatase-treated ribosomal RNA's of *Bacillus stearothermophilus.* Heavy (23*S*) and light (16*S*) RNA fractions were prepared from the ribosomal RNA of *Bacillus stearothermophilus,* and half of each was treated with alkaline phosphatase. The intact and phosphatase-treated RNA fractions were incubated with (γ^{32}P)-ATP and polynucleotide kinase, as described in ref 1. The respective reaction mixtures were passed through Sephadex G200 columns and the RNA fractions eluted from the columns were placed on sucrose density gradients formed in Spinco SW25 tubes. After centrifugation for 20 hr at 25,000 rpm and 5°C, the radioactivity and OD_{260} profiles were determined.

RNA's of *B. stearothermophilus* are shown in Figure 1. The amount of *P transferred to the 16*S* RNA fraction reached about 1.3 mμ moles/mg RNA ($OD_{260}^{1\%} = 24$) and that to the 23*S* RNA fraction about 0.75 mμmole/mg RNA. These amounts correspond to about 70–80 per cent of the termini as calculated from the molecular weights.[8] It was concluded that the ribosomal RNA's of various organisms were phosphorylated at the 5′-termini, as in the case of *E. coli* ribosomal RNA. Accordingly, the terminal phosphoryl groups of the RNA's were removed by treatment with alkaline phosphatase and the 5′-termini were rephosphorylated with *P using polynucleotide kinase. The phosphorylated RNA was reisolated from the reaction mixture by passing through Sephadex G200 columns and by sucrose-density-gradient centrifugation. The radioactivity peak region at which the ratio of the radioactivity to OD_{260} was almost constant was collected and hydrolyzed with alkali, and with T1 and pancreatic RNases. The alkaline hydrolysate was chromatographed on Dowex 1 columns to identify *pYp (Y = C, U, A, or G). The radioactive fragments produced by T1 or pancreatic RNase treatment were separated by chromatography on DEAE-Sephadex columns to determine the chain length of the fragments. The results obtained are summarized in Table 2.

The ribosomal RNA's from *B. subtilis, B. cereus, and B. stearothermophilus* gave very similar results. The alkaline hydrolysates of two ribosomal RNA's both produced *pUp predominantly, showing that the major component of these RNA's had uridine at the 5′-termini. The T1 RNase hydrolysate of the heavy RNA component produced a main radioactivity peak at the region corresponding to *pXpXpXpXpXpGp. When this fraction was collected and hydrolyzed with pancreatic RNase, more than 80 per cent of the radioactivity was found at the *pPyp region. This was identified as *pUp by Dowex 1 column chromatography. Since the pancreatic RNase hydrolysate of the original 5′-(*P)-RNA also produced predominantly *pUp, it was concluded that the major component of the heavy ribosomal RNA of the three *Bacilli* had the sequence *pUpXpXpXpXpGp, at the 5′-terminus. By a similar procedure, it was found that the terminus of the major component of the light ribosomal RNA was *pUpXpXpXpXpXpGp, because T1 RNase and pancreatic RNase hydrolysis produced *pXpXpXpXpXpXpGp and *pUp, respectively, and pancreatic RNase treatment of the *pXpXpXpXpXpXpGp fragment produced *pUp predominantly.

The pancreatic and T1 RNase hydrolysates of the heavy RNA component of *S. lutea* had major radioactive fragments at the regions corresponding to *pPupPupPupPupPyp and *pXpXpGp, respectively. Since alkaline hydrolysis of the original 5′(*P)-RNA produced *pAp predominantly, the terminal sequence was concluded to be *pApApGpPyp. This was confirmed by T1-RNase treatment of the *pPupPupPupPyp fragment which produced *pApApGp. In contrast, T1 RNase hydrolysis of the light RNA component produced a main peak of radioactivity at *pXpXpXpXpXpGp. The peak position was shifted to the *pPyp region by pancreatic RNase treatment of this fragment. As the alkaline and pancreatic RNase hydrolysates both produced predominantly *pUp, it was concluded that the major component of the light ribosomal RNA of *S. lutea* started with pUpXpXpXpXpGp.

The major radioactive fragments produced from the light RNA component of yeast by pancreatic and T1 RNase hydrolysis were *pUp and *pXpXpXpXpXpXpGp, respectively. Since alkaline hydrolysis also gave predominantly *pUp, it was con-

TABLE 2

RADIOACTIVE FRAGMENTS PRODUCED FROM 5′(P^{32})-RIBOSOMAL RNA'S BY ALKALINE, T1 RNASE AND PANCREATIC RNASE HYDROLYSIS

Bacillus cereus

	Alkaline Hydrolysis (% of total)			T1 RNase Hydrolysis (% recovery)			Pancreatic RNase Hydrolysis (% recovery)	
	Heavy RNA	Light RNA		Heavy RNA	Light RNA		Heavy RNA	Light RNA
*pCp	3.0	4.4	*pGp	4.6	6.8	*pPyp	*83.8*	*71.5*
*pAp	9.6	13.2	*pXpGp	7.8	8.9	*pPupPyp	4.3	7.8
*pGp	2.5	1.6	*pXpXpGp	8.2	5.4	*pPupPupPyp	3.8	4.0
*pUp	*85.0*	*81.0*	*pXpXpXpGp	3.0	4.0	*pPupPupPupPyp	2.8	2.4
			*pXpXpXpXpGp	*70.5*	13.7	*pPupPupPupPupPyp	1.3	1.6
			*pXpXpXpXpXpGp	1.9	*53.6*	*pPupPupPupPupPupPyp	0.9	1.2

Bacillus subtilis

	Alkaline Hydrolysis (% of total)			T1 RNase Hydrolysis (% recovery)			Pancreatic RNase Hydrolysis (% recovery)	
	Heavy RNA	Light RNA		Heavy RNA	Light RNA		Heavy RNA	Light RNA
*pCp	2.7	9.8	*pGp	11.2	4.4	*pPyp	*73.7*	*70.6*
*pAp	23.0	22.0	*pXpGp	14.8	7.9	*pPupPyp	8.5	13.0
			*pXpXpGp	7.0	4.3	*pPupPupPyp	8.5	5.3
*pGp	3.4	2.2	*pXpXpXpGp	2.6	5.5	*pPupPupPupPyp	5.6	3.0
*pUp	*71.0*	*66.0*	*pXpXpXpXpGp	*62.2*	4.5	*pPupPupPupPupPyp	2.2	1.2
			*pXpXpXpXpXpGp	1.5	*63.2*	*pPupPupPupPupPupPyp	1.6	0.7

Bacillus stearothermophilus

	Alkaline Hydrolysis (% of total)			T1 RNase Hydrolysis (% recovery)			Pancreatic RNase Hydrolysis (% recovery)	
	Heavy RNA	Light RNA		Heavy RNA	Light RNA		Heavy RNA	Light RNA
*pCp	3.8	2.2	*pGp	10.1	5.4	*pPyp	*65.8*	*70.0*
*pAp	21.8	10.7	*pXpGp	19.2	8.1	*pPupPyp	8.5	3.8
			*pXpXpGp	7.7	2.8	*pPupPupPyp	9.9	4.5
*pGp	3.5	1.1	*pXpXpXpGp	3.3	1.6	*pPupPupPupPyp	7.3	1.4
*pUp	*70.9*	*85.7*	*pXpXpXpXpGp	*57.7*	6.6	*pPupPupPupPupPyp	5.0	1.7
			*pXpXpXpXpXpGp	1.1	*64.8*	*pPupPupPupPupPupPyp	1.8	0.8

Sarcina lutea

	Alkaline Hydrolysis			T1 RNase Hydrolysis			Pancreatic RNase Hydrolysis	
	Heavy RNA (% of total)	Light RNA (% of total)		Heavy RNA (% recovery)	Light RNA (% recovery)		Heavy RNA (% recovery)	Light RNA (% recovery)
*pCp	2.4	3.1	*pGp	10.3	3.4	*pPyp	21.0	*85.0*
*pAp	*82.0*	10.8	*pXpGp	12.6	5.3	*pPupPyp	9.9	4.8
			*pXpXpGp	*61.7*	4.4	*pPupPupPyp	7.8	3.0
*pGp	2.8	2.5	*pXpXpXpGp	3.1	20.7	*pPupPupPupPyp	*57.5*	3.9
*pUp	12.7	*83.6*	*pXpXpXpXpGp	4.9	*64.6*	*pPupPupPupPupPyp	1.4	1.8
			*pXpXpXpXpXpGp	1.6	1.1	*pPupPupPupPupPupPyp	0.4	1.5

Saccharomyces cerevisiae

	Alkaline Hydrolysis			T1 RNase Hydrolysis			Pancreatic RNase Hydrolysis	
	Heavy RNA (% of total)	Light RNA (% of total)		Heavy RNA (% recovery)	Light RNA (% recovery)		Heavy RNA (% recovery)	Light RNA (% recovery)
*pCp	1.9	6.0	*pGp	6.7	6.7	*pPyp	*58.0*	*76.8*
*pAp	*31.6*	11.1	*pXpGp	8.5	8.5	*pPupPyp	8.1	6.4
			*pXpXpGp	*56.3*	4.1	*pPupPupPyp	4.6	5.4
*pGp	9.6	4.0	*pXpXpXpGp	1.3	2.6	*pPupPupPupPyp	*24.6*	2.9
*pUp	*57.0*	*84.5*	*pXpXpXpXpGp	1.6	1.8	*pPupPupPupPupPyp	2.7	2.1
			*pXpXpXpXpXpGp	3.7	*70.3*	*pPupPupPupPupPupPyp	0.9	1.7

X = C, A, or U.

Procedures for preparation of 5′(P^{32})-ribosomal RNA's were described in the legend to Fig. 1. Aliquots were hydrolyzed with alkali (0.3 *N* KOH, 37°C for 20 hr). A mixture of nonradioactive ADP, CDP, GDP, and UDP was added to the hydrolysate and the mixture was chromatographed on Dowex 1X2 columns to separate *pAp, *pCp, *pGp, and *pUp. To the remainder, nonradioactive RNA was added to furnish UV markers and the mixture was hydrolyzed either with pancreatic RNase or T1 RNase. The resulting radioactive fragments were analyzed by chromatography on DEAE-Sephadex columns.

TABLE 3

COMPARISON OF THE 5′-TERMINAL NUCLEOTIDE SEQUENCES OF THE MAJOR COMPONENTS CONTAINED IN EACH CLASS OF RIBOSOMAL RNA'S FROM SIX DIFFERENT ORGANISMS

Organisms	Heavy RNA	Light RNA
Bacillus cereus	pUpXpXpXpGp-----	pUpXpXpXpXpGp----
Bacillus subtilis	pUpXpXpXpGp-----	pUpXpXpXpXpGp----
Bacillus stearothermophilus	pUpXpXpXpGp-----	pUpXpXpXpXpGp----
*Escherichia coli**	pGpGpUp----------	pApApApUpGp--------
Sarcina lutea	pApApGpPyp-------	pUpXpXpXpGp-------
Saccharomyces cerevisiae	pUpUpGp----------	pUpXpXpXpXpGp----

* Taken from refs. 1 and 6.

cluded that the terminus of the light RNA component of yeast was *pUpXpXpX-pXpGp. In contrast, T1 RNase hydrolysis of the heavy RNA fraction of yeast produced predominantly *pXpXpGp. Pancreatic RNase treatment of this fragment gave *pUp with good recovery, so that the sequence was shown to be *pUp-XpGp. The second base was further identified by chromatography on a Dowex 1 column, according to the procedure in the *Methods* section, and it was found that the sequence was *pUpUpGp.

As expected, the pancreatic RNase hydrolysate of the original 5′(*P)-RNA yielded *pUp as the main fragment. However, it was noted in this analysis that a second significant peak of radioactivity was at the pPupPupPupPyp region (see Table 2). The chromatographic position of this peak was not affected by T1 RNase treatment, so it was assumed to be *pApApApPyp. The fourth Py base was identified as cytosine by column chromatography.[6] In accordance with these observations, the alkaline hydrolysate showed the presence of a significant amount (about 32%) of pAp in addition to the main *pUp (57%). Therefore, it appeared that the heavy RNA fraction of yeast contained some RNA fraction which started with a sequence of pApApApCp in addition to the major component of which the terminus was pUpUpGp. With respect to this additional component, no significant second radioactivity peak was found until in the hexamer region in the analysis of the T1 RNase hydrolysate. The fact suggests that the position of the first G from the 5′-end of the second component is located further inside the chain, beyond the first six residues.

The terminal sequences obtained from the above analyses are summarized in Table 3, compared with the data for two *E. coli* ribosomal RNA's. The termini listed in Table 2 are those of the main components of the respective ribosomal RNA's. Recovery of the radioactivity in those main components was around 60–70 per cent of the total phosphorylated termini. The components which were the next in amount to the major component were analyzed and were found to be distributed in a number of other fragments, as in the case of *E. coli* ribosomal RNA's.[1] The only exception so far was the heavy RNA fraction of yeast, in which the second component was clearly identified.

Discussion.—It was found that the major component of *E. coli* 16*S* ribosomal RNA started with AAAUG-- and that of the 23*S* ribosomal RNA with GGU--.[1, 6] We have now analyzed the 5′-termini of ribosomal RNA's of five different organisms and have shown that the main components of these RNA's also start with unique sequences, differing in various organisms. Midgley compared the relationship between the nucleotide base compositions of DNA and RNA of a variety of orga-

nisms and reported that the nucleotide composition of their ribosomal RNA's was very similar but that the GC/AT ratios of DNA varied widely[2] (see also Table 1). Our present data show the sequences of only a few nucleotides starting at the 5′-end and do not give information on any possible homology of the remainder of the molecules. However, our observations clearly indicate that the molecular species of ribosomal RNA's differ in various organisms. In this connection, it is noteworthy that hydrolysis of ribosomal RNA's of three species of the genus *Bacillus* produced fragments that were identical in length and in the 5′-terminal base. The observations suggest that the molecular species of ribosomal RNA within the same genus exhibit taxonomic similarity.

The recovery of radioactivity in the major components of the ribosomal RNA's averaged about 60 to 70 per cent of the total present in phosphorylated termini (Table 2). Oligonucleotides which were present in addition to the major component were examined, and the balance of the radioactivity was scattered through a number of other fragments, except the case of the heavy ribosomal RNA component of yeast. Since RNA of high molecular weight is extremely fragile, there is a strong probability that some of the ribosomal RNA molecules were cleaved near the 5′-terminal region during the preparation or enzymatic treatment of ribosomal RNA, that these partially degraded molecules became phosphorylated with *P, and that they were present in the fractions analyzed. The fractions may also have contained degraded fragments of messenger RNA. With these assumptions the main radioactive fragments identified are presumed to be the authentic termini of ribosomal RNA's and other, minor, fragments are background, although we cannot rule out an alternate interpretation that each ribosomal RNA is heterogeneous and contains a number of molecules which start with sequences differing from those of its major component.

Summary.—The 5′-terminal nucleotide sequences of ribosomal RNA's from one yeast and four bacterial species were analyzed by aid of a method based on an enzymatic phosphorylation of the terminal 5′-hydroxyl group. All ribosomal RNA's examined were found to be phosphorylated at the 5′-termini, so they were first treated with alkaline phosphatase to remove the terminal phosphoryl group, and then rephosphorylated with highly radioactive orthophosphate by using polynucleotide kinase. They were then hydrolyzed by alkali, T1 RNase, and pancreatic RNase, and the resulting radioactive fragments were analyzed. Each of the ribosomal RNA's examined showed considerable homogeneity with respect to the 5′-termini, and the major components in each class of ribosomal RNA's started with unique, but different, sequences.

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